RESEARCH PAPER

Epigenetics 8:6, 635–645; June 2013; © 2013 Landes Bioscience

Epigenetic and transcriptional features of the novel human imprinted lncRNA *GPR1AS* suggest it is a functional ortholog to mouse *Zdbf2linc*

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Keywords: lncRNA, genomic imprinting, DNA methylation, antisense RNA, placenta

Long non-coding RNAs (lncRNAs), transcribed from the intergenic regions of animal genomes, play important roles in key biological processes. In mice, *Zdbf2linc* was recently identified as an lncRNA isoform of the paternally expressed imprinted *Zdbf2* gene. The functional role of *Zdbf2linc* remains undefined, but it may control parent-of-origin-specific expression of protein-coding neighbors through epigenetic modification in cis, similar to imprinted *Nespas*, *Kcnq1ot1* and *Airn* lncRNAs. Here, we identified a novel imprinted long-range non-coding RNA, termed *GPR1AS*, in the human *GPR1-ZDBF2* intergenic region. Although *GPR1AS* contains no human *ZDBF2* exons, this lncRNA is transcribed in the antisense orientation from the *GPR1* intron to a secondary, differentially methylated region upstream of the *ZDBF2* gene (*ZDBF2* DMR), similar to mouse *Zdbf2linc*. Interestingly, *GPR1AS/Zdbf2linc* is exclusively expressed in human/mouse placenta with paternal-allele-specific expression and maternal-allele-specific promoter methylation (*GPR1/Gpr1* DMR). The paternal-allele specific methylation of the secondary *ZDBF2* DMR was established in human placentas as well as somatic lineage. Meanwhile, the *ZDBF2* gene showed stochastic paternal-allele-specific expression, possibly methylation-independent, in placental tissues. Overall, we demonstrated that epigenetic regulation mechanisms in the imprinted *GPR1-GPR1AS-ZDBF2* region were well-conserved between human and mouse genomes without the high sequence conservation of the intergenic lncRNAs. Our findings also suggest that lncRNAs with highly conserved epigenetic and transcriptional regulation across species arose by divergent evolution from a common ancestor, if they do not have identical exon structures.

Introduction

One surprising result of the human genome project is the discovery that protein coding sequences account for a very small fraction (~1%) of the genomic sequence.[1] Human transcriptome analysis revealed that only one-fifth of transcription across the genome is associated with protein-coding genes.[2] The untranslated transcripts are broadly referred to as "non-coding" RNA molecules and include small RNAs and long non-coding RNAs (lncRNAs). Small RNAs (e.g., miRNA, piRNA, and snoRNA) are critically important in a variety of biological processes.[3] However, small RNAs represent a small portion of the non-coding genes; the majority of non-coding RNAs are lncRNAs.[2] The lncRNAs are defined as non-coding RNAs with more than 200 nucleotides. Genome-wide experimental and computational studies have shown that lncRNAs are poorly conserved across species; for instance, only 14% of the mouse lncRNAs have human orthologs.[4,5] Although they generally are less well conserved across species and often show high tissue specificity and low expression levels across multiple tissues and developmental stages, studies have revealed that lncRNAs also play important roles in gene regulation through chromatin remodeling, e.g., X chromosome dosage compensation or regulation of *HOX* genes.[3,6-8] Nonetheless, most lncRNAs remain poorly understood; this class of RNA has become a new paradigm for gene regulation.

In placental mammals, the functional non-equivalence of the parental genomes is mediated by "genomic imprinting," an epigenetic mechanism by which imprinted genes are expressed in a parent-of-origin-specific manner.[9] Approximately 150 imprinted genes have been identified so far in humans and mice, and the imprinting status of many is conserved across mammalian species (imprinted gene catalogs: www.mousebook.org/catalog.php?catalog=imprinting, igc.otago.ac.nz/home.html, www.geneimprint.com). Most of the imprinted genes occur in clusters with silencing controlled by an imprint control region (ICR) that is methylated on one parental allele, a stable epigenetic mark established during gametogenesis.[10] The unmethlyated ICR acts as promoter for lncRNAs such as *Nespas*, *Kcnq1ot1,* and *Airn*,

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Submitted: 03/19/13; Revised: 04/25/13; Accepted: 05/01/13
http://dx.doi.org/10.4161/epi.24887

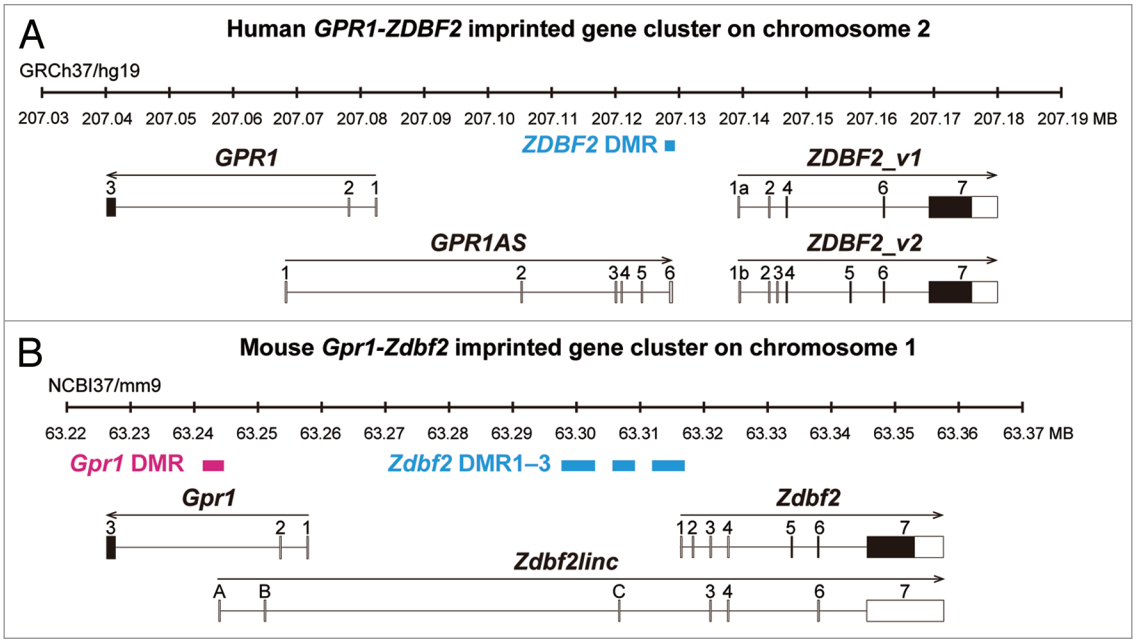

Figure 1. Schematic physical maps of human (top) and mouse (bottom) orthologous imprinted *GPR1-GPR1AS-ZDBF2/Gpr1-Zdbf2linc-Zdbf2* clusters. Imprinted genes are represented as follows: Open and filled boxes represent untranslated regions and ORFs, respectively; arrows indicate transcription orientation. Human *GPR1AS* does not contain any significant ORFs. *ZDBF2_v2* codes for an ORF similar to the original ZDBF2 protein sequence encoded by *ZDBF2_v1* (see **Fig. S1**). Magenta and cyan bars represent maternally or paternally methylated DMRs in mice. The extent of each DMR was determined in previous DNA methylome studies.[20-22]

which then silence all imprinted genes in the cluster on that parental allele.[11-17] Thus, these imprinted gene clusters provide important epigenetic models for the numerous lncRNAs mapped in the mammalian genome. We and other groups have identified an imprinted gene cluster containing the *GPR1*/*Gpr1* (G protein-coupled receptor 1) and *ZDBF2*/*Zdbf2* (zinc finger, DBF-type containing 2) genes on the human chromosome 2q3.33 and mouse chromosome 1C2; both are specifically expressed from the paternal allele.[18-20] *Zdbf2* exhibits imprinted expression in various fetal and adult tissues, whereas *Gpr1* exhibits kidney-specific imprinted expression in mice. Furthermore, we identified a novel mouse *Zdbf2* isoform, *Zdbf2linc*, a long-range (transcription across 114 kb) non-coding RNA, transcribed from *Gpr1* intron 2 in the antisense orientation with respect to *Gpr1* (**Fig. 1**).[23] *Zdbf2linc* is also expressed in a paternal-allele-specific manner and, importantly, both the non-coding and original transcripts were controlled by maternal DNA methylation imprints via DNMT3L, a cofactor of DNA methyltransferase.[24] The imprinted *Gpr1-Zdbf2* locus therefore has two similarities with the above-referenced imprinted clusters: (1) two differential states of imprinted DNA methylation, namely, maternal-allele-specific methylation at ICRs (also called "primary" differentially methylated regions or DMRs) and paternal-allele-specific methylation established after fertilization/implantation at "secondary" DMRs, and (2) paternal specific transcription of lncRNAs from ICRs regulated by maternal methylation.[23] Although little is known about the rule of these secondary DMRs, their monoallelic methylation marks may have essential regulatory function for the single or multiple imprinted neighbor genes during post-fertilization development.[25] Mouse imprinted *Gpr1-Zdbf2* domain harbors the germline-derived primary *Gpr1* DMR at the promoter of *Zdbf2linc* and the post-fertilization-derived secondary *Zdbf2* DMR1–3 upstream of the *Zdbf2* gene (**Fig. 1**). In human, only *ZDBF2* DMR, which exhibits paternal-allele-specific DNA methylation, was identified at the intergenic region between *GPR1* and *ZDBF2*.[21] We must elucidate the transcriptional and epigenetic profiles and structures of a gene cluster to understand the mechanisms by which imprinted expression is regulated.

In this study, we identified a novel, long (> 1.5 kb, transcription across 60 kb) imprinted, non-coding gene transcribed from *GPR1* intron 2 in the human genome. The novel lncRNA gene does not include any human *ZDBF2* exons (meaning that the transcript is not an isoform of human *ZDBF2*), but its expression profile is very similar to that of mouse *Zdbf2linc*. Our findings strongly indicate that epigenetic and transcriptional regulation mechanisms of the *GPR1-ZDBF2* locus are well-conserved between human and mouse genomes and that this lncRNA might play a crucial role in local gene regulation.

Results

Identification of novel long-range RNA transcript, *GPR1AS*, at the human *GPR1-ZDBF2* locus. We mapped the 5' end of the human *ZDBF2* transcript using rapid amplification of cDNA ends (RACE) analysis of human embryonic stem (ES) cells to identify a human *ZDBF2* splicing variant homologous to the mouse *Zdbf2linc*. The original and new variants were

termed *ZDBF2_v1* and *ZDBF2_v2*. *ZDBF2_v1* contains 5 original exons, renamed exon 1a, 2, 4, 6 and 7 (RefSeq database: exon 1–5). *ZDBF2_v2* contains an alternative first exon, exon 1b, located between the first and second exons of *ZDBF2_v1*; 4 exons match NCBI exons (RefSeq database: exon 2–5); extra exons (exon 3 and 5) are located between the second/third and third/fourth exons of *ZDBF2_v1* (**Fig. 1**; **Table S1**). *ZDBF2_v2* has a large open reading frame (ORF, 2352 amino acids), similar to the ZDBF2 protein encoded by *ZDBF2_v1* (**Fig. S1**). It is unclear whether the newly identified *ZDBF2_v2* is the true coding or non-coding RNA; however, we identified no large intergenic isoform of *ZDBF2* transcribed from the *GPR1* gene (as well as mouse *Zdbf2linc*) in this approach.

Next, we focused on an EST clone, BE734382 (914 bp without the poly-A tail), which is an antisense transcript transcribed from *GPR1* intron 2 to the *ZDBF2* gene, just like the mouse *Zdbf2linc* (**Fig. S2**). To determine the full length of the antisense *GPR1* gene transcript, we therefore performed 5'- and 3'-RACE using human ES cells. The RACE analyses showed the antisense transcript encodes a 1505-nt RNA molecule and contains 6 exons; the first exon is located at *GPR1* intron 2 and the remaining 5 exons are located in the intergenic region between *GPR1* and *ZDBF2* (**Fig. 1**; **Fig. S3**). This spliced antisense RNA is transcribed through the 60-kb genomic region but does not include any *ZDBF2* exons and contains no large ORFs encoding larger than 100 amino acids. Interestingly, the transcription termination site of this novel "lncRNA" reaches and overlaps the human *ZDBF2* DMR, the known DMR upstream of the *ZDBF2* gene.[21] We named this novel gene encoding a long antisense non-coding RNA transcript *GPR1-AntiSense* (*GPR1AS*).

***GPR1AS* is a novel paternally expressed imprinted gene in the human genome.** Next, we characterized *GPR1AS/Zdbf2linc* expression profiles in fetal and adult human, and mouse tissues by relative quantification in real-time RT-PCR. Therefore, we demonstrated that *GPR1AS/Zdbf2linc* is exclusively and strongly expressed in the placenta and adult testis, and *Zdbf2linc* is expressed weakly in mouse brain; however, *GPR1AS/Zdbf2linc* transcript levels were very low or almost undetectable in other tissues (**Fig. 2**). Notably, this strong placental expression of *GPR1AS* is reminiscent of the original BE734382 EST clone obtained from human placenta. We also performed allelic expression profiling of human placentas using single nucleotide polymorphism (SNP). Three SNPs have been identified in *GPR1AS* exons; we designed RT-PCR primers within these exons to detect these SNPs (reference SNP ID: rs1024809; at exon 2, rs34523400 and rs10932150; at exon 6). Allele-specific expression analysis was performed by direct sequencing of RT-PCR products of these exons in informative cases: four human placental tissues from a child heterozygous for individual SNP sites, and only the paternal allele was detected (**Fig. 3**). Thus, we demonstrated that *GPR1AS* is a novel imprinted gene that is expressed from a paternal allele in the human placenta.

Our previous study in mice demonstrated that *Zdbf2* and *Zdbf2linc* transcripts were controlled by maternal methylation imprinting, most likely in the *Gpr1* DMR at the promoter region of *Zdbf2linc*.[23] Therefore, we investigated the methylation

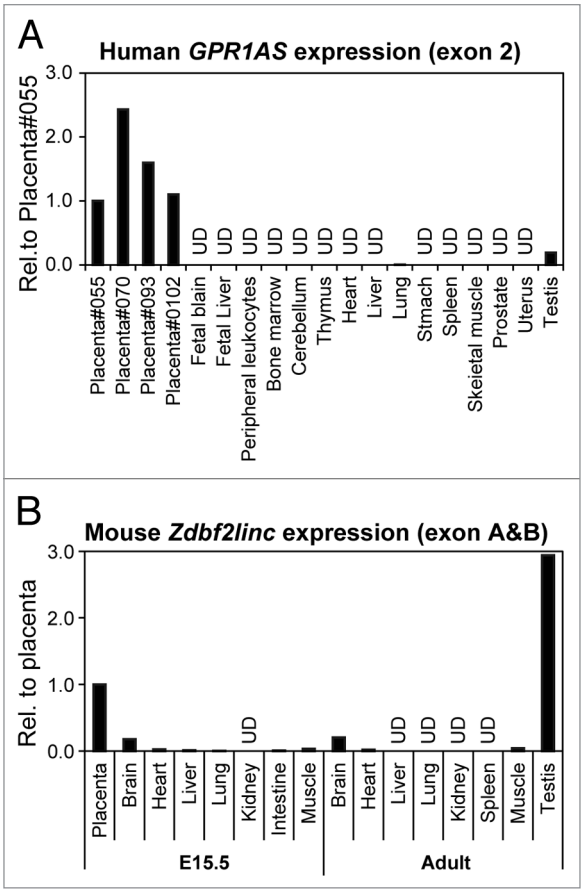

Figure 2. Relative expression of *GPR1AS/Zdbf2linc* in multiple human and mouse tissues (qRT-PCR). Expression in the human placenta (Family ID: 055) or mouse placenta at E15.5 is set as 1 for each gene. UD means undetectable after 40 cycles of amplification.

status of the promoter region of *GPR1AS* in human placental tissues. Three SNPs have been identified upstream of the first *GPR1AS* exon (reference SNP ID: rs16838070, rs1683074 and rs130009940). Of these, rs16838070 and rs1683074 were observed in the Japanese population, and were in strong linkage disequilibrium; rs130009940 is rare Japanese (data not shown). Therefore, we designed primer sets for bisulfite sequencing within the promoter region that includes rs1683074. Four informative cases of placentas and umbilical cord blood lymphocytes were investigated. In all cases, the *GPR1AS* promoter was hypomethylated (methylation levels, ≤ 25%) on the active paternal allele and hypermethylated (methylation levels, ≥ 75%) on the repressed maternal allele in placenta; the promoter was hypermethylated on both parental alleles in blood lymphocytes (**Fig. 4**). Thus, a novel maternally methylated "human *GPR1* DMR" was identified in a human region orthologous to the mouse *Gpr1* DMR.

Imprinted *GPR1AS/Zdbf2linc* gene expression with parent-of-origin-specific methylation was retained in extra-embryonic lineages. Placental imprinted expression of human *GPR1AS* suggests mouse *Zdbf2linc* may also be expressed in an allele-specific manner in placental tissues. Previously, we demonstrated that *Zdbf2linc* expression is paternally expressed

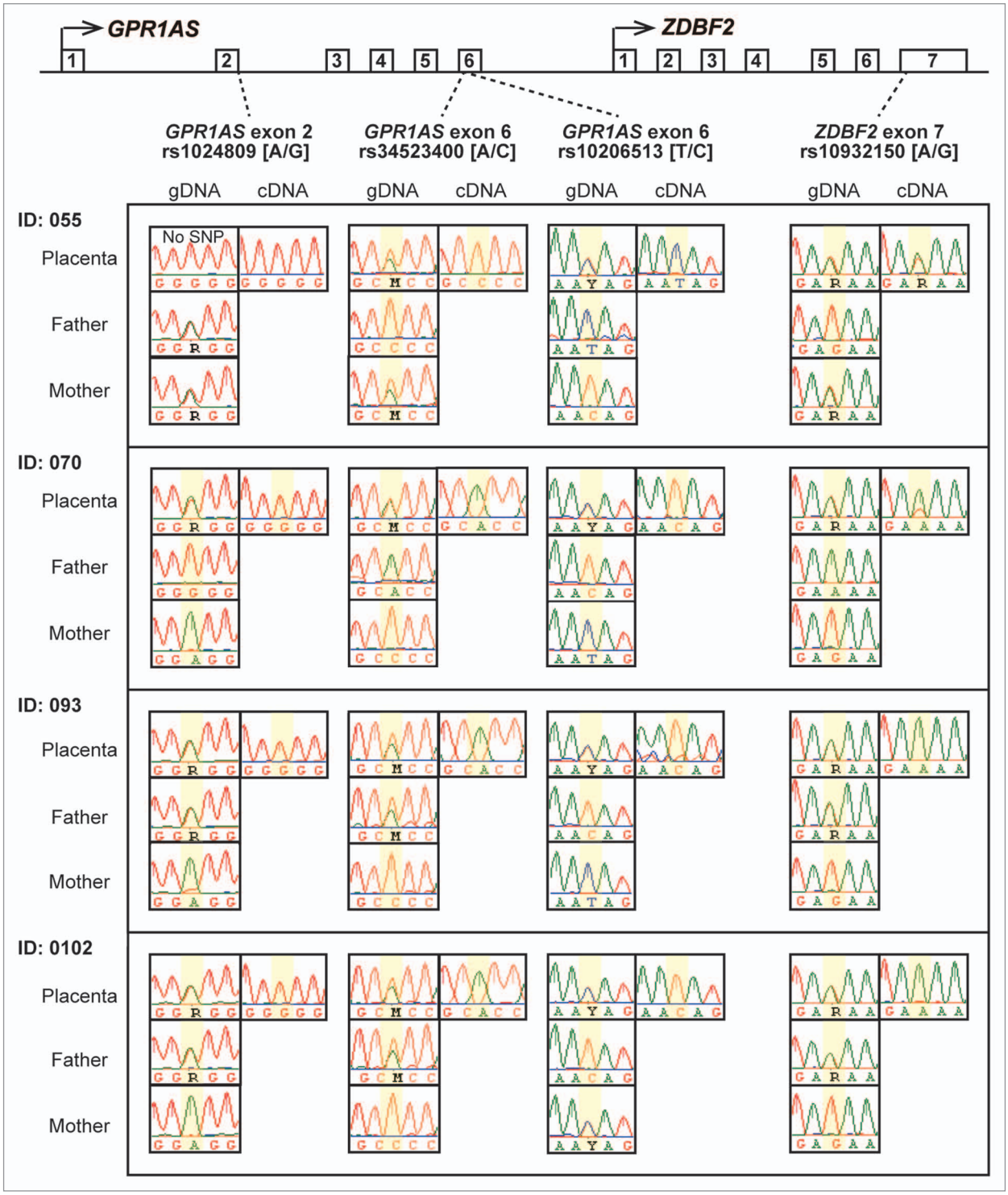

Figure 3. Allele-specific RT-PCR sequencing of *GPR1AS* and *ZDBF2* in human placentas. Four heterozygous genotypes were used to distinguish between maternal and paternal alleles in human placental samples from four Japanese patients (National Center for Child Health and Development). SNP positions are highlighted in yellow.

during the blastula to gastrula stages in mouse embryos, but is rarely expressed post-gastrulation, with bi-allelic complete methylation at the *Gpr1* DMR.[23] However, the expression profiles were not investigated in extra-embryonic tissues. Therefore, we re-analyzed *Zdbf2linc* expression profiles in mouse E3.5 to E9.5 embryonic tissues, including extra-embryonic tissues, by quantitative real-time RT-PCR (qRT-PCR). The accumulation of *Zdbf2linc* mRNA in whole embryos or embryonic and extra-embryonic tissues was clearly detected during blastula (E3.5) and gastrula (E5.5 to E7.5) stages; however, although

Zdbf2linc mRNA levels were very low or undetectable in embryos, it remained detectable in ectoplacental cones (placental precursors) post-gastrulation (E9.5) (**Fig. 5A**). We performed allele-specific expression analysis of mouse tissues, including placental tissues, in which *Zdbf2linc* expression was detectable, in a C57BL/6N × JF1 genetic background (designated B6 and JF) using SNP information. Predictably, *Zdbf2linc* showed paternal-allele-specific expression in placental tissues at E9.5 and E15.5 (**Fig. 5B**). Thus, as with human *GPR1AS*, imprinted expression of *Zdbf2linc* appeared in placental tissues. Meanwhile, paternal-allele-specific expression was also observed in the fetal brain at E9.5, whereas bi-parental expression was observed in adult testis (**Fig. S4**). Testis-specific escape from imprinting has been also observed for *Zdbf2* exon 7.[19]

To characterize the correlation between imprinted expression of *Zdbf2linc* and methylation status of the promoter region of *Zdbf2linc* (*Gpr1* DMR), we compared the methylation status of *Gpr1* DMR in mouse embryonic and placental tissues at E9.5. Interestingly, the placental tissues showed maternal-allele-specific methylation, whereas embryonic tissues showed hypermethylation of both parental alleles in the *Gpr1* DMR (**Fig. 6**). Our previous study showed the *Gpr1* DMR exhibits maternal-allele-specific methylation during blastula to gastrula stages in mice. Thus, our previous and current data showed that *GPR1AS*/*Zdbf2linc* is rarely expressed throughout post-gastrulation development and into somatic tissues due to bi-allelic hypermethylation at their transcription start sites.

Stochastic paternal-allele-specific expression of *ZDBF2*/*Zdbf2* in placental lineages. In mice, *Zdbf2* transcription is positively correlated with methylation of the *Zdbf2* DMRs (*Zdbf2* DMR1–3), established on the paternal allele in the post-implantation embryo, upstream of *Zdbf2*.[23] In human, as previously mentioned, paternally methylated DMR (*ZDBF2* DMR) was also identified at intergenic region between *GPR1* and *ZDBF2*.[21] We investigated the methylation status of the *ZDBF2* DMR in human placental tissues and umbilical cord blood lymphocytes. A SNP has been identified at *ZDBF2* DMR (reference SNP ID: rs10206513), but any SNP has not been found around the first exon of *ZDBF2*. Therefore, we designed primer sets for bisulfite sequencing within the *ZDBF2* DMR, which includes rs10206513, and the *ZDBF2* promoter region. Four informative cases of placentas and lymphocytes were investigated; these cases have also been investigated in **Figure 3**. Interestingly, both placental tissues and lymphocytes showed clear maternal-allele-specific hypermethylation in the *ZDBF2* DMR and almost complete hypomethylation in the *ZDBF2* promoter (**Fig. 7**).

We previously described bi-allelic *ZDBF2* expression in human placental tissues after allelic expression profiling of coding exon (exon 7); however, the study included only one human placenta.[19] Here, we re-analyzed allelic the expression profiles of *ZDBF2* in human placental tissues. Similar to the *GPR1AS* gene, we performed allele-specific expression analysis of *ZDBF2* in human placentas (reference SNP ID: rs10932150, at *ZDBF2* exon 7). Four placentas were investigated and we were surprised to find only one case (Family ID: 055) showed bi-allelic expression; the other three cases showed paternal-allele-specific expression

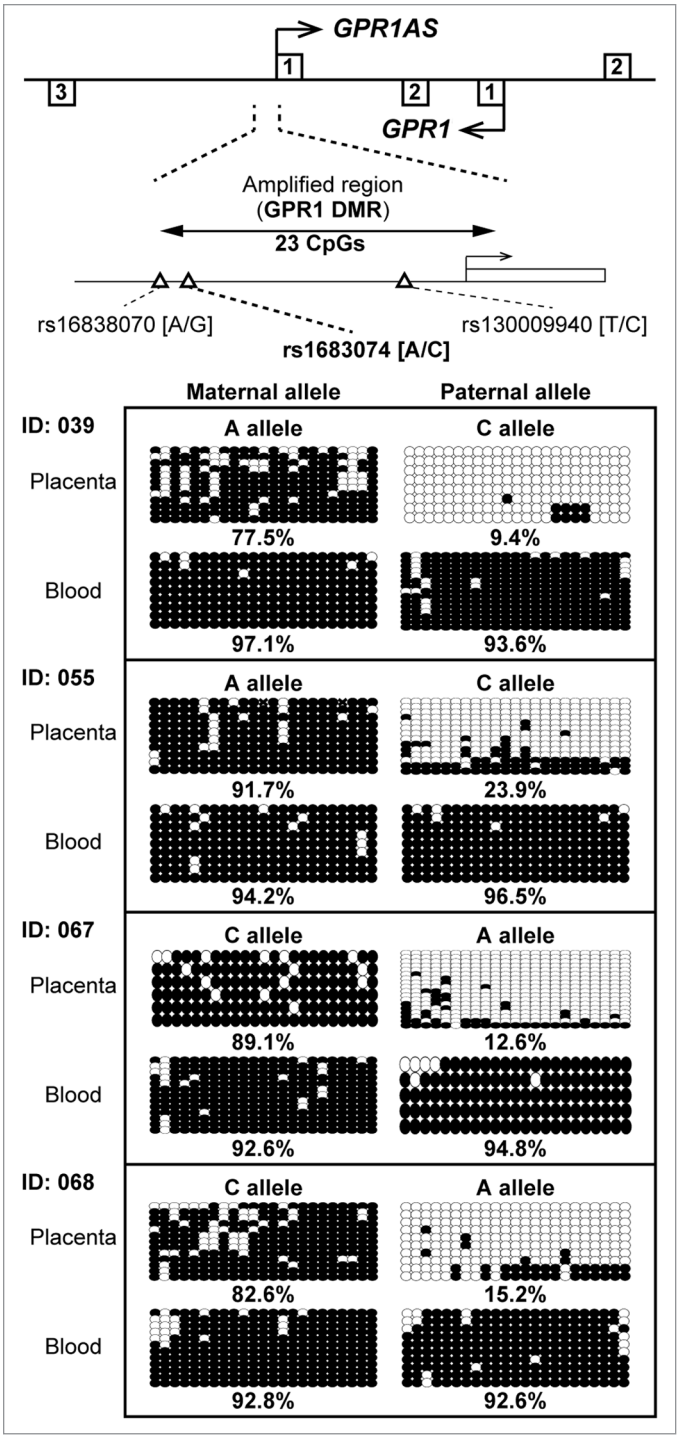

Figure 4. Bisulfite sequencing of the *GPR1AS* promoter region in human placenta and umbilical blood samples. Each row represents the results of an independent sequencing reaction. Open and closed circles denote unmethylated and methylated CpGs. Calculated methylation levels are shown below each graph. Heterozygous SNP rs1683074 was used to distinguish between maternal and paternal alleles in human samples. One of case (Family ID: 055) was also investigated by allelic expression analysis in **Figure 3**.

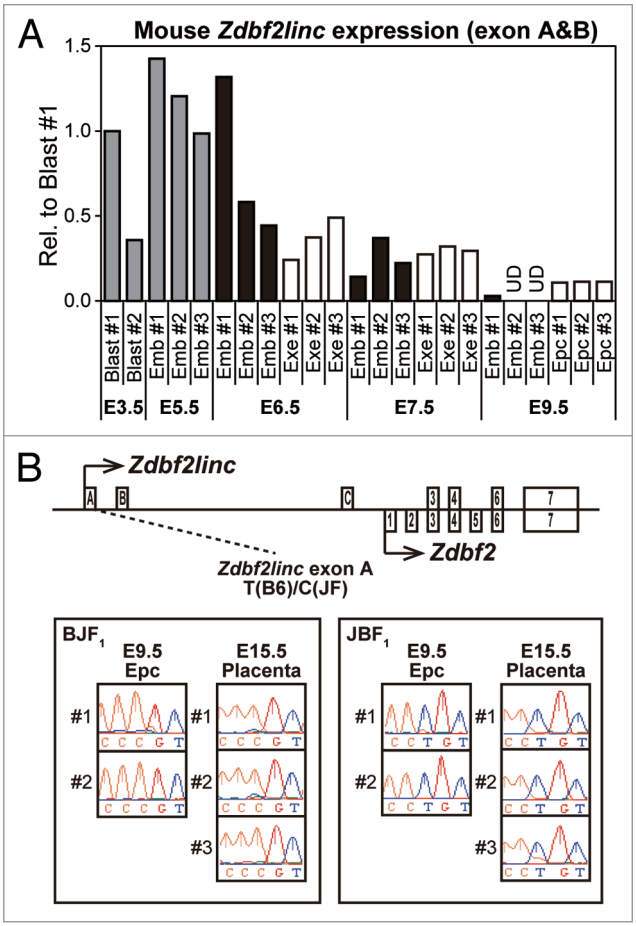

Figure 5. (**A**) Embryonic and extra-embryonic expression of *Zdbf2linc* in pre- and post-gastrulation mouse embryo (at E3.5 to E9.5). Abbreviations: E3.5 Blast, pools of 5 blastocysts (gray bars); E5.5 Emb, whole embryos (gray bars), E6.5-E9.5, dissected embryos free of extra-embryonic tissues (black bars); E6.5-E7.5 Exe, extra-embryonic tissues (white bars); E9.5 Epc, ectoplacental cones dissected from the remaining extra-embryonic tissues (white bars). After normalization against the housekeeping *Actb* gene, relative expression in mouse blast #1 is set as 1. UD indicates undetected mRNA expression. (**B**) Allele-specific RT-PCR sequencing of *Zdbf2linc*-specific exon (left) and common *Zdbf2* exon (right) in mouse ectoplacental cones at E9.5 and placentas at E15.5. We prepared 2 parental mouse strains, C57BL/6N (B6) and JF1/Msf (JF), and reciprocal F_1 hybrids (BJF_1; F_1 hybrid derived from mating between B6 female and JF male mice, JBF_1; the reciprocal cross of JF female with B6 male mice).

(**Fig. 3**). Although it is possible that maternal contamination of fetal tissue obscures parental expression bias of *ZDBF2* in some placental cases (e.g., Family ID: 055 in human case),[26,27] our results indicated stochastic, paternal-allele-specific *ZDBF2* expression in placental tissues and that imprinting might be independent of DNA methylation at the *ZDBF2* DMR or *ZDBF2* promoter.

Mouse germ cell DNA methylome profiling in the *Gpr1-Zdbf2* locus. We previously performed whole-genome shotgun bisulfite sequencing of samples from various stages of mouse germ cells, blastocysts and ES cells.[22,28] To clear the hotspot for DNA methylation, we generated and investigated the DNA methylome map of mouse *Gpr1-Zdbf2* (**Fig. S5**). In sperm cells, although almost CpGs including *Zdbf2* DMRs were hypermethylated, all promoters and CpG islands (including *Gpr1* DMR, *Gpr1* promoter and *Zdbf2* CpG island promoter) were hypomethylated. The paternally methylated *Zdbf2* DMRs were demethylated (probably biallelically) in blastocysts. This is consistent with the fact that *Zdbf2* DMRs were secondary DMRs, in which paternal-allele-specific methylation was re-established during post-implantation fetal development.[23] In oocytes, the intragenic region of *Gpr1*, including the *Gpr1* DMR, was hypermethylated, whereas the other intergenic and intragenic regions were hypomethylated. All CpG islands except *Gpr1* DMR remained hypomethylated during early embryo development and gametogenesis. Complete hypomethylation of the *Zdbf2linc* promoter (*Gpr1* DMR) might result in bi-allelic expression of *Zdbf2linc* in adult testes containing spermatogenetic cells.

Comparison of human and mouse *GPR1-ZDBF2*/*Gpr1-Zdbf2* loci. These results indicated that epigenetic and transcriptional profiles of human *GPR1AS* and mouse *Zdbf2linc* were similar. Our findings and those of previous studies demonstrated that the regulatory mechanism of the imprinted *GPR1-ZDBF2* cluster was well-conserved between human and mouse. Therefore, we used the advanced PipMaker program to perform a detailed comparison between mouse and human genomic sequences. This analysis is shown graphically in the percent identity plot (PIP) in **Figure 8** for the protein-coding exons of *GPR1* and *ZDBF2*. Overall, the PIP revealed strong conservation of *GPR1*/*Gpr1* and *ZDBF2*/*Zdbf2* between the human and mouse genomes and poor conservation of *GPR1AS* lncRNAs, with a number of transposable elements (**Fig. S6**). Evolutionary conservation is also supported by certain similarities between their human/mouse gene products (GPR1 proteins, 94% similarity; ZDBF2 proteins, 76% similarity) (**Fig. S7**). In all our comparisons, the exon sequences and structures of human *GPR1AS* were very different from the mouse *Zdbf2linc* transcript.

Discussion

We identified a novel antisense lncRNA gene, *GPR1AS*, at the imprinted *GPR1-ZDBF2* domain on human chromosome 2q33.3. *GPR1AS* is specifically expressed in placenta and testis; in the placenta, the paternal allele is exclusively expressed. We identified several similarities between human *GPR1AS* and mouse *Zdbf2linc* lncRNAs: (1) antisense transcription from *GPR1*/*Gpr1* intron 2; (2) long intergenic transcripts (human *GPR1AS*, 60 kb; mouse *Zdbf2linc*, 114 kb) throughout the secondary *ZDBF2*/*Zdbf2* DMRs; (3) specific expression in ES cells, placental tissues, and testis; (4) transcript expression from the paternal allele with silencing of the maternal allele by promoter DNA hypermethylation in placental tissues; and (5) complete biallelic promoter methylation and silencing in fetal and adult tissues (**Fig. 9**). These facts strongly suggested that *GPR1AS* is a human ortholog of mouse *Zdbf2linc*. Furthermore, previous studies have revealed that neighbor genes, *GPR1*/*Gpr1* and *ZDBF2*/*Zdbf2*, are also commonly imprinted with maternal and paternal DMRs (*GPR1*/*Gpr1* and *ZDBF2*/*Zdbf2* DMRs) in human and mouse.

These and previous findings demonstrated that epigenetic and transcriptional regulatory mechanisms of the imprinted *GPR1-ZDBF2* cluster were well conserved between human and mouse genomes. Some structural differences were also identified: (1) no *ZDBF2* exon in the *GPR1AS* transcript and (2) no significant similarity between lncRNA sequences. However, the conservation of epigenetic and transcriptional traits between *GPR1AS* and *Zdbf2linc* can be assumed to have arisen by divergent evolution from a common ancestor, in spite of their differing sequences, and are likely functional.

Imprinted gene clusters in the eutherian genome contain many lncRNA genes, most of which are imprinted. These non-coding RNAs are often closely linked to the regulation of imprinted expression in *cis*. Perhaps the best studied of such molecules are the imprinted *Nespas*, *Kcnq1ot*1 and *Airn* lncRNAs. These lncRNAs induce parental-specific silencing and suggest their transcription is more important than their products.[13-15,17] The mono-allelic transcription of *GPR1AS*/*Zdbf2linc* lncRNAs may have a similar cis-regulating effect on flanking coding genes because these lncRNAs were transcribed from an unmethylated ICR candidate (maternally methylated primary *GPR1*/*Gpr1* DMR: There is no direct evidence that this region actually acts as an ICR because there is no report of a knockout mouse) as well as the three functional imprinted lncRNAs. Some studies have suggested that transcription across DMRs is associated with epigenetic changes and acquisition of genomic imprinting.[13,29-31] For example, Williamson et al. demonstrated that paternally expressed *Nespas* lncRNA transcription is required for demethylation of trimethylated histone H3 lysine 4, followed by DNA methylation, at the secondary *Nesp* DMR.[13] Despite the differing exon structures of *GPR1AS* and *Zdbf2linc*, both lncRNA transcripts reached the secondary *ZDBF2*/*Zdbf2* DMRs (**Figs. 1 and 9**). It is also possible that monoallelic epigenetic modification (e.g., paternal-allele-specific methylation of *ZDBF2*/*Zdbf2* DMRs) is established via long-range transcription of *GPR1AS* and *Zdbf2linc* during the early embryonic stage, and such epigenetic modification might stably control the parental-allele expression of *ZDBF2*/*Zdbf2* in fetal and adult somatic tissues, even after the disappearance of lncRNA transcript (**Fig. 9**).[23] Although paternal-allele-specific methylation at *ZDBF2* DMRs was established in all tested cases, stochastic non-imprinted expression of *ZDBF2* was observed in placental tissues. Perhaps for this reason, imprinted expression of *ZDBF2* may be independent of DNA methylation in placental lineages, similar to the imprinted *Kcnq1* gene clusters.[32] In both human and mice, promoter regions of *ZDBF2*/*Zdbf2* were completely hypomethylated whereas their paternal-allele-specific expression (**Fig. 7**; **Fig. S5**). Thus, the promoter might be marked monoallelically by other epigenetic modification (e.g., histone modifications). In mice, we previously described bi-allelic *Zdbf2* expression in (E15.5) placentas by expression profiling of exon 7 (18); however, this exon is common between *Zdbf2* and *Zdbf2linc*. Unfortunately, between B6 and JF mice, no SNPs were detectable in coding *Zdbf2*-specific exons 1, 2 and 5 (data not shown); therefore, further analysis is required to determine whether coding *Zdbf2* variant is expressed in a parent-of-origin-specific manner in placental tissues, e.g., using other mouse strains.

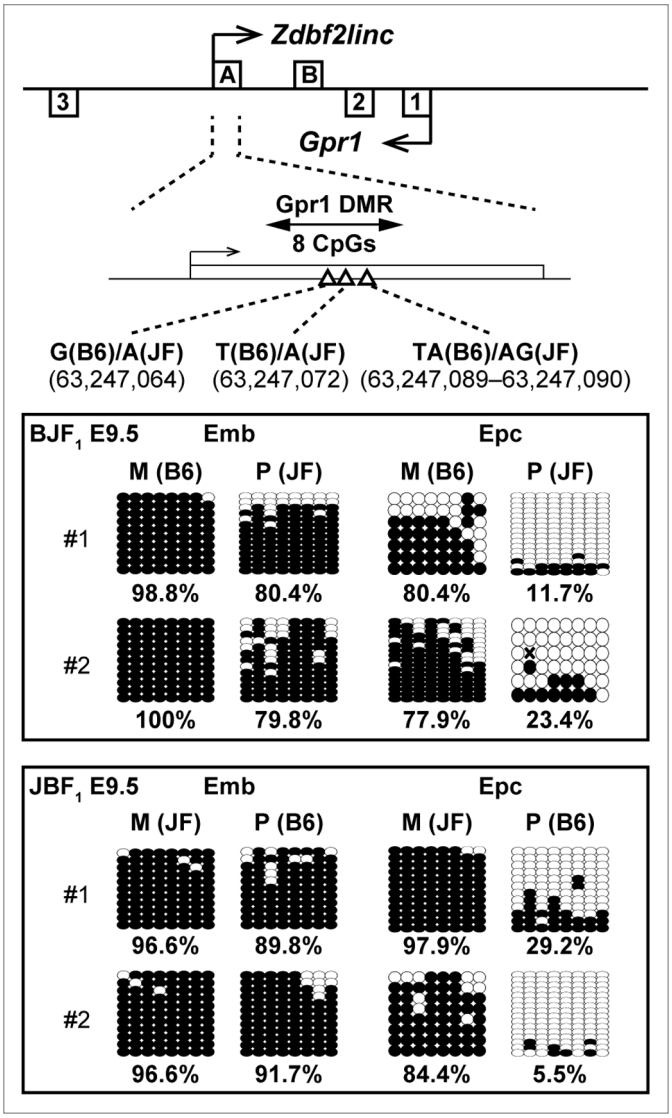

Figure 6. Bisulfite sequencing of *Gpr1* DMR in mouse BJF_1/JBF_1 embryos and placental tissues at E9.5. M and P indicate maternally and paternally inherited alleles. Both alleles were discriminated by more than one polymorphism.

In mammals, over a thousand evolutionarily conserved long-intergenic non-coding RNAs (lincRNAs: lncRNAs overlapping intergenic regions) have been identified by their chromatin structures.[33] Although it is now estimated that the human genome produces over 8000 lincRNAs, only ~12% of human lincRNAs have orthologous transcripts in other species and only a few of them have been functionally characterized.[34] These functional lincRNAs play essential roles in the regulation of epigenetic marks and gene expression in trans or cis. For example, *HOTAIR* lincRNA silences transcription across 40 kb of the *HOXD* locus by inducing a repressive chromatin state in trans,[35] and the *XIST*, *TSIX*, *JPX*, and *XACT* lincRNAs are key players in X-chromosome inactivation (XCI) in cis.[36-39] The XCI and genomic imprinting represent prominent examples of the recruitment of chromatin-modifying activities by lncRNAs and provides a prototype model

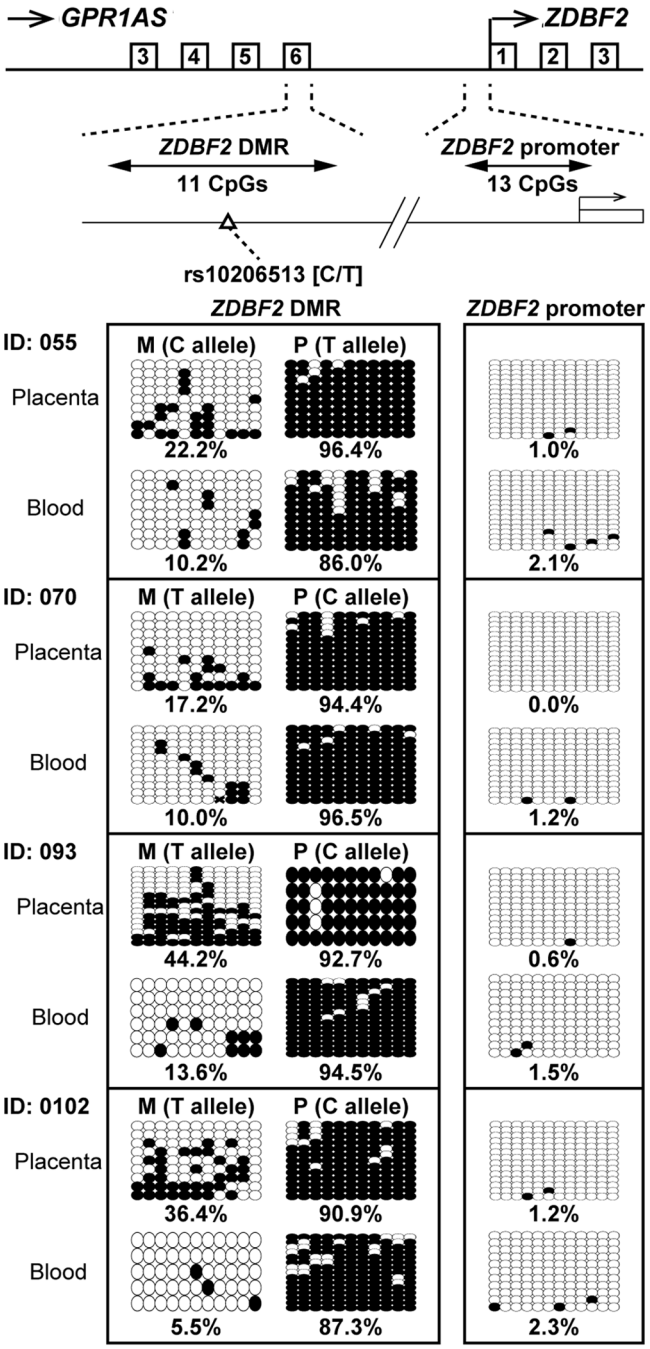

Figure 7. Bisulfite sequencing of *ZDBF2* DMR and *ZDBF2* promoter in human placenta and umbilical blood samples. Calculated methylation levels are shown below the graphs. Heterozygous SNP rs10206513 was used to distinguish between maternal and paternal alleles in human samples (all cases were tested in **Figure 3**).

for a cis localization mechanism. Our findings suggested the most important cue for cis-acting lncRNA might be transcription through a particular region (e.g., cis-regulating elements, such as promoter, silencer and insulator) via specific interaction with chromatin to mediate targeted recruitment of repressive epigenetic-modifying activities to silence transcription. Thus, the lncRNA sequences and structures might not be important. Our results suggest many more homologous lncRNAs could be defined using transcriptional and epigenetic profiles including DNA methylation. lncRNAs represent a largely unexplored world of genetic/epigenetic variation and may provide mechanistic insight into transcriptional regulation.

Materials and Methods

Human samples. All human studies were approved by the Institutional Review Board Committee at the National Center for Child Health and Development (NCCHD) and performed after obtaining written informed consent. The derivation and cultivation of human ES Cell (hESC) lines was performed in full compliance with "The Guidelines on the Derivation and Distribution of Human Embryonic Stem Cells" of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (Notification No.156 of 2009). We used MEXT-registered hESC lines, SEES1 and SEES3.Genomic DNA from human placentas was isolated with a standard proteinase K-phenol/chloroform procedure. Genomic DNA from human lymphocytes was isolated with the QIAamp DNA Blood Midi Kit (Qiagen). Total RNA from human placentas was extracted with Sepasol-RNA I Super G (Nacalai Tesque); total RNA from hESCs was extracted with the Allprep DNR/RNA Mini Kit (Qiagen). RNA was treated with RQ1 RNase-free DNase (Promega) and purified by phenol/chloroform extraction. Total RNA from other human normal tissues were purchased from TaKaRa Bio.

Mouse samples. Mouse embryos, placental tissues, fetal tissues, and adult tissues were prepared as described previously.[19,23,40] To use DNA polymorphisms for allele discrimination, reciprocal F_1 hybrids were obtained by crossing C57BL/6N (B6; Clea Japan) and JF1/Msf (JF; National Institute of Genetics) mice. Total RNA from E3.5–7.5 embryos and extra-embryonic tissues were extracted with the RNeasy Mini Kit. Genomic DNA and Total RNA were isolated from E9.5 embryos and ectoplacental cones (yolk sacs and amnions removed) with the Allprep DNR/RNA Mini Kit (Qiagen). Total RNA from E15.5 placental and fetal tissues and adult tissues from 8-week-old male mice were isolated with TRIzol reagent (Invitrogen). The extracted RNA was treated with RQ1 RNase-free DNase (Promega) and purified by phenol/chloroform extraction.

RACE analysis. The 5'- and 3'-regions of the Human *ZDBF2* and *GPR1AS* gene was obtained using the GeneRacer Kit (Invitrogen). Genomic DNA-free total RNA was prepared from human ES cells and 2 rounds of PCR were performed using TaKaRa EX Taq (TaKaRa Bio) under the following conditions: 5 cycles of 30 sec at 94°C, 1 min at 72°C, 5 cycles of 30 sec at 94°C, 1 min at 70°C, 20 cycles of 30 sec at 94°C, 30 sec at 64°C, 1 min at 68°C and 10 min final elongation at 68°C for the first PCR; and 20 cycles of 30 sec at 94°C, 30 sec at 65°C, 2 min at 68°C and 10 min final elongation at 68°C for the second PCR. The *Zdbf2* gene-specific primers for the nested PCR were as follows: anti-sense *ZDBF2* 5RA1: 5'-GCA TTA CAA ATC ACT GGG GGG CGT ACC A-3' for the first PCR and anti-sense *ZDBF2* 5RA2: 5'-CTG GGG GGC GTA CCA AGT TGC TTC TA-3' for the

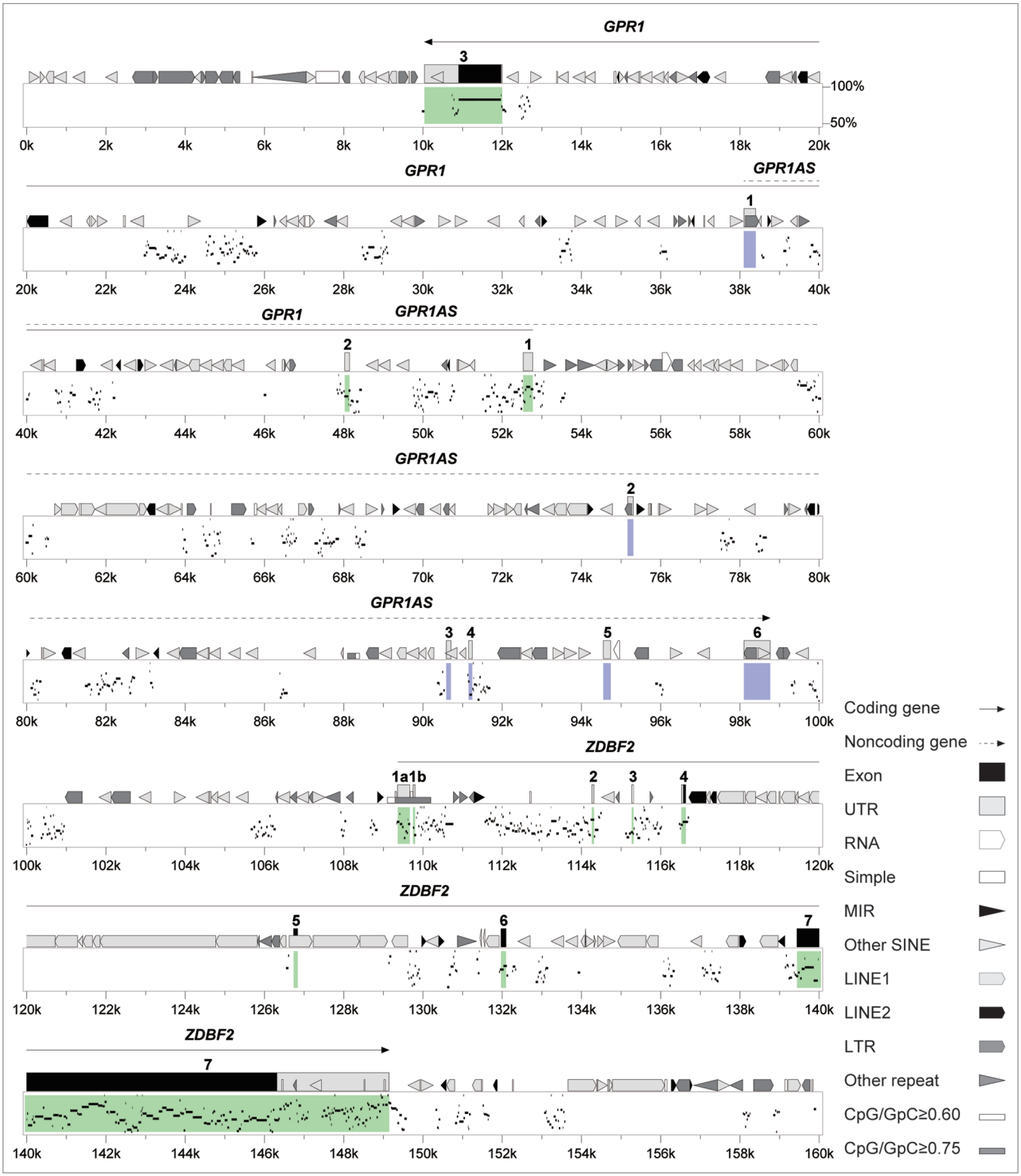

Figure 8. Human and mouse sequences of the imprinted *GPR1-ZDBF2* domain. Percent Identity Plot (PIP, a scale of 50% to 100% conservation, on the *y*-axis) showing order and alignment of the entire imprinted domain on human chromosome 2q33.3 (chr2: 207,030,001–207,190,000, on the *x*-axis) and the orthologous region on mouse chromosome 1C2. Detected sequence homology (> 50% identity) is plotted by position along the test sequence and level of sequence similarity. Structural features in the human ortholog, including exons, repeats and CpG-rich regions, are shown above the top line. The novel imprinted lncRNA gene, *GPR1AS*, is shown in blue, and known imprinted protein-coding genes, *GPR1* and *ZDBF2*, are shown in green.

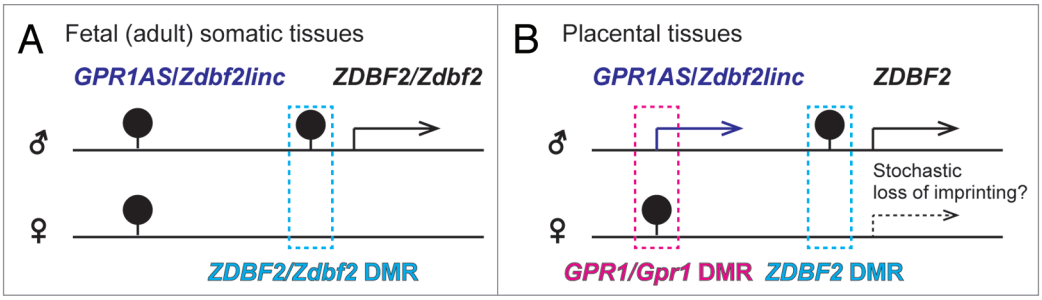

Figure 9. Summary of allele-specific epigenetic and gene expression differences within the *GPR1AS-ZDBF2* (*Zdbf2linc-Zdbf2*) locus during fetal (**A**) and extra-embryonic (**B**) development. Arrows represent transcriptional orientation of individual imprinted genes. DMR positions are indicated by filled pins on the methylated alleles.

second PCR for 5'RACE. The BE734382 (*GPR1AS* gene) primer sets nested PCR were as follows: anti-sense BE734382_5RA1: 5'-TAC TAC AAG GGC CAG AAC TCA GGA AGA G-3' for the first PCR and anti-sense BE734382_5RA2: 5'-TGG AAG CGA TGC ACG GGA CAA GGT CT-3' for the second PCR for 5'RACE, and sense BE734382_3RA1: 5'-AGG AGC CCC AGA GGG CCA TTG TAA TTC-3' for the first PCR and sense BE734382_3RA2: 5'-TCA AGA TCT TCA GTA AAC TTC TGC CGC-3' for the second PCR for 3'RACE. Spliced form of *GPR1AS* was obtained by PCR using two sets of primers; BE734382_F1: 5'-GTC CCG TGC ATC GCT TCC AT-3' and BE734382_R1: 5'-CCA CCT TTT TCC AAG TGG CC-3', and BE734382_F2: 5'-ATC CGG CTC TTC CTG AGT TC-3' and BE734382_R2: 5'-CGG CAG AAG TTT ACT GAA GA-3'. The amplified products were sequenced directly, after purification.

Real-time RT-PCR and allelic expression analysis. Genomic DNA-free total RNA samples from human and mouse fetal samples were reverse transcribed to cDNA with SuperScript III (Invitrogen). Quantitative expression analysis of human *GPR1AS* and mouse *Zdbf2linc* was performed in a 7500 Real-time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). Relative expression of each gene was calculated by the ΔΔCt method and normalized to human *ACTB* or mouse *Actb* housekeeping genes. RT-PCR and direct sequencing of *GPR1AS*/*Zdbf2linc* and *ZDBF2*/*Zdbf2* exons were performed using an ABI PRISM 3730xl genetic analyzer (Applied Biosystems) as described previously.[19] Primer sequences and PCR cycling conditions are listed in **Table S2**.

Bisulfite sequencing. Genomic DNA samples were isolated from human samples and mouse E9.5 embryos and placental tissues from an F_1 hybrid of B6 and JF. Genomic DNA (100 ng per sample) was treated with sodium bisulfite using the MethylCode Bisulfite Conversion Kit (Invitrogen). The bisulfite-treated DNA was PCR-amplified with TaKaRa EpiTaq HS (TaKaRa Bio) with primers specific for human *GPR1* DMR, *ZDBF2* DMR, *ZDBF2* promoter and mouse *Gpr1* DMR. Primers and PCR cycling conditions are listed in **Table S2**. Subcloning and sequencing were performed as described.[19] Visualization and quantification of bisulfite sequence data for CpG methylation was performed using the QUMA web-based tool.[41]

Comparative in silico analysis. The genomic alignments of *GPR1* and *ZDBF2* were obtained from the UCSC Genome Browser database (genome.ucsc.edu) for human (GRCh37/hg19) and mouse (NCBI37/mm9) genomes. The Advanced PipMaker program (pipmaker.bx.psu.edu/cgi-bin/pipmaker?advanced) was used to compare the sequences of the genomic regions surrounding human *GPR1* and *ZDBF2* (hg19, chr2: 207,030,001–207,190,000) and mouse *Gpr1* and *Zdbf2* (mm9, chr1: 63,160,001–63,380,000). Repetitive DNA sequences were classified using RepeatMasker (www.repeatmasker.org). The nucleotide and amino acid sequences were aligned by Genetyx software (Genetyx Corporation).

Accession numbers. Full-length sequences of human *GPR1AS* and *ZDBF2* and mouse *Zdbf2linc* have been deposited DNA Data Bank of Japan (DDBJ) under accession numbers AB774455, AB775780, AB775781 and AB777270.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to thank Dr. Akihiro Umezawa and other members of NCCHD for providing hESCs and for many helpful discussions. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (Grant No. 222228004) awarded to T.K., including the MEXT-Supported Program for the Strategic Research Foundation at Private Universities (Grant No. S0801025). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/24887

References

1. Birney E, Stamatoyannopoulos JA, Dutta A, Guigó R, Gingeras TR, Margulies EH, et al.; ENCODE Project Consortium; NISC Comparative Sequencing Program; Baylor College of Medicine Human Genome Sequencing Center; Washington University Genome Sequencing Center; Broad Institute; Children's Hospital Oakland Research Institute. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 2007; 447:799-816; PMID:17571346; http://dx.doi.org/10.1038/nature05874
2. Kapranov P, Willingham AT, Gingeras TR. Genome-wide transcription and the implications for genomic organization. Nat Rev Genet 2007; 8:413-23; PMID:17486121; http://dx.doi.org/10.1038/nrg2083
3. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12:861-74; PMID:22094949; http://dx.doi.org/10.1038/nrg3074
4. Church DM, Goodstadt L, Hillier LW, Zody MC, Goldstein S, She X, et al.; Mouse Genome Sequencing Consortium. Lineage-specific biology revealed by a finished genome assembly of the mouse. PLoS Biol 2009; 7:e1000112; PMID:19468303; http://dx.doi.org/10.1371/journal.pbio.1000112
5. Chodroff RA, Goodstadt L, Sirey TM, Oliver PL, Davies KE, Green ED, et al. Long noncoding RNA genes: conservation of sequence and brain expression among diverse amniotes. Genome Biol 2010; 11:R72; PMID:20624288; http://dx.doi.org/10.1186/gb-2010-11-7-r72
6. Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS. Specific expression of long noncoding RNAs in the mouse brain. Proc Natl Acad Sci U S A 2008; 105:716-21; PMID:18184812; http://dx.doi.org/10.1073/pnas.0706729105
7. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136:629-41; PMID:19239885; http://dx.doi.org/10.1016/j.cell.2009.02.006
8. Guttman M, Donaghey J, Carey BW, Garber M, Grenier JK, Munson G, et al. lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature 2011; 477:295-300; PMID:21874018; http://dx.doi.org/10.1038/nature10398
9. Reik W, Walter J. Genomic imprinting: parental influence on the genome. Nat Rev Genet 2001; 2:21-32; PMID:11253064; http://dx.doi.org/10.1038/35047554
10. Sasaki H, Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 2008; 9:129-40; PMID:18197165; http://dx.doi.org/10.1038/nrg2295
11. Sleutels F, Zwart R, Barlow DP. The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature 2002; 415:810-3; PMID:11845212; http://dx.doi.org/10.1038/415810a
12. Mancini-Dinardo D, Steele SJ, Levorse JM, Ingram RS, Tilghman SM. Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes Dev 2006; 20:1268-82; PMID:16702402; http://dx.doi.org/10.1101/gad.1416906
13. Williamson CM, Ball ST, Dawson C, Mehta S, Beechey CV, Fray M, et al. Uncoupling antisense-mediated silencing and DNA methylation in the imprinted Gnas cluster. PLoS Genet 2011; 7:e1001347; PMID:21455290; http://dx.doi.org/10.1371/journal.pgen.1001347
14. Latos PA, Pauler FM, Koerner MV, Şenergin HB, Hudson QJ, Stocsits RR, et al. Airn transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. Science 2012; 338:1469-72; PMID:23239737; http://dx.doi.org/10.1126/science.1228110
15. Mohammad F, Pandey GK, Mondal T, Enroth S, Redrup L, Gyllensten U, et al. Long noncoding RNA-mediated maintenance of DNA methylation and transcriptional gene silencing. Development 2012; 139:2792-803; PMID:22721776; http://dx.doi.org/10.1242/dev.079566
16. Williamson CM, Turner MD, Ball ST, Nottingham WT, Glenister P, Fray M, et al. Identification of an imprinting control region affecting the expression of all transcripts in the Gnas cluster. Nat Genet 2006; 38:350-5; PMID:16462745; http://dx.doi.org/10.1038/ng1731
17. Korostowski L, Sedlak N, Engel N. The Kcnq1ot1 long non-coding RNA affects chromatin conformation and expression of Kcnq1, but does not regulate its imprinting in the developing heart. PLoS Genet 2012; 8:e1002956; PMID:23028363; http://dx.doi.org/10.1371/journal.pgen.1002956
18. Babak T, Deveale B, Armour C, Raymond C, Cleary MA, van der Kooy D, et al. Global survey of genomic imprinting by transcriptome sequencing. Curr Biol 2008; 18:1735-41; PMID:19026546; http://dx.doi.org/10.1016/j.cub.2008.09.044
19. Kobayashi H, Yamada K, Morita S, Hiura H, Fukuda A, Kagami M, et al. Identification of the mouse paternally expressed imprinted gene Zdbf2 on chromosome 1 and its imprinted human homolog ZDBF2 on chromosome 2. Genomics 2009; 93:461-72; PMID:19200453; http://dx.doi.org/10.1016/j.ygeno.2008.12.012
20. Hiura H, Sugawara A, Ogawa H, John RM, Miyauchi N, Miyanari Y, et al. A tripartite paternally methylated region within the Gpr1-Zdbf2 imprinted domain on mouse chromosome 1 identified by meDIP-on-chip. Nucleic Acids Res 2010; 38:4929-45; PMID:20385583; http://dx.doi.org/10.1093/nar/gkq200
21. Fang F, Hodges E, Molaro A, Dean M, Hannon GJ, Smith AD. Genomic landscape of human allele-specific DNA methylation. Proc Natl Acad Sci U S A 2012; 109:7332-7; PMID:22523239; http://dx.doi.org/10.1073/pnas.1201310109
22. Kobayashi H, Sakurai T, Imai M, Takahashi N, Fukuda A, Yayoi O, et al. Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish oocyte-specific heritable marks. PLoS Genet 2012; 8:e1002440; PMID:22242016; http://dx.doi.org/10.1371/journal.pgen.1002440
23. Kobayashi H, Sakurai T, Sato S, Nakabayashi K, Hata K, Kono T. Imprinted DNA methylation reprogramming during early mouse embryogenesis at the Gpr1-Zdbf2 locus is linked to long cis-intergenic transcription. FEBS Lett 2012; 586:827-33; PMID:22449967; http://dx.doi.org/10.1016/j.febslet.2012.01.059
24. Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development 2002; 129:1983-93; PMID:11934864
25. Kagami M, O'Sullivan MJ, Green AJ, Watabe Y, Arisaka O, Masawa N, et al. The IG-DMR and the MEG3-DMR at human chromosome 14q32.2: hierarchical interaction and distinct functional properties as imprinting control centers. PLoS Genet 2010; 6:e1000992; PMID:20585555; http://dx.doi.org/10.1371/journal.pgen.1000992
26. Hudson QJ, Seidl CI, Kulinski TM, Huang R, Warczok KE, Bittner R, et al. Extra-embryonic-specific imprinted expression is restricted to defined lineages in the post-implantation embryo. Dev Biol 2011; 353:420-31; PMID:21354127; http://dx.doi.org/10.1016/j.ydbio.2011.02.017
27. Okae H, Hiura H, Nishida Y, Funayama R, Tanaka S, Chiba H, et al. Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression. Hum Mol Genet 2012; 21:548-58; PMID:22025075; http://dx.doi.org/10.1093/hmg/ddr488
28. Kobayashi H, Sakurai T, Miura F, Imai M, Mochiduki K, Yanagisawa E, et al. High-resolution DNA methylome analysis of primordial germ cells identifies gender-specific reprogramming in mice. Genome Res 2013; 23:616-27; PMID:23410886; http://dx.doi.org/10.1101/gr.148023.112
29. Chotalia M, Smallwood SA, Ruf N, Dawson C, Lucifero D, Frontera M, et al. Transcription is required for establishment of germline methylation marks at imprinted genes. Genes Dev 2009; 23:105-17; PMID:19136628; http://dx.doi.org/10.1101/gad.495809
30. Henckel A, Chebli K, Kota SK, Arnaud P, Feil R. Transcription and histone methylation changes correlate with imprint acquisition in male germ cells. EMBO J 2012; 31:606-15; PMID:22117218; http://dx.doi.org/10.1038/emboj.2011.425
31. Smith EY, Futtner CR, Chamberlain SJ, Johnstone KA, Resnick JL. Transcription is required to establish maternal imprinting at the Prader-Willi syndrome and Angelman syndrome locus. PLoS Genet 2011; 7:e1002422; PMID:22242001; http://dx.doi.org/10.1371/journal.pgen.1002422
32. Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, et al. Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet 2004; 36:1291-5; PMID:15516931; http://dx.doi.org/10.1038/ng1468
33. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009; 458:223-7; PMID:19182780; http://dx.doi.org/10.1038/nature07672
34. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev 2011; 25:1915-27; PMID:21890647; http://dx.doi.org/10.1101/gad.17446611
35. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007; 129:1311-23; PMID:17604720; http://dx.doi.org/10.1016/j.cell.2007.05.022
36. Tian D, Sun S, Lee JT. The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation. Cell 2010; 143:390-403; PMID:21029862; http://dx.doi.org/10.1016/j.cell.2010.09.049
37. Sado T, Brockdorff N. Advances in understanding chromosome silencing by the long non-coding RNA Xist. Philos Trans R Soc Lond B Biol Sci 2013; 368:20110325; PMID:23166390; http://dx.doi.org/10.1098/rstb.2011.0325
38. Ohhata T, Senner CE, Hemberger M, Wutz A. Lineage-specific function of the noncoding Tsix RNA for Xist repression and Xi reactivation in mice. Genes Dev 2011; 25:1702-15; PMID:21852535; http://dx.doi.org/10.1101/gad.16997911
39. Vallot C, Huret C, Lesecque Y, Resch A, Oudrhiri N, Bennaceur-Griscelli A, et al. XACT, a long noncoding transcript coating the active X chromosome in human pluripotent cells. Nat Genet 2013; 45:239-41; PMID:23334669; http://dx.doi.org/10.1038/ng.2530
40. Sato S, Yoshida W, Soejima H, Nakabayashi K, Hata K. Methylation dynamics of IG-DMR and Gtl2-DMR during murine embryonic and placental development. Genomics 2011; 98:120-7; PMID:21620950; http://dx.doi.org/10.1016/j.ygeno.2011.05.003
41. Kumaki Y, Oda M, Okano M. QUMA: quantification tool for methylation analysis. Nucleic Acids Res 2008; 36(Web Server issue):W170-5; PMID:18487274; http://dx.doi.org/10.1093/nar/gkn294